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Phospholipase Cδ regulates germination of *Dictyostelium* spores

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Abstract

Background: Many eukaryotes, including plants and fungi make spores that resist severe environmental stress. The micro-organism *Dictyostelium* contains a single phospholipase C gene (PLC); deletion of the gene has no effect on growth, cell movement and differentiation. In this report we show that PLC is essential to sense the environment of food-activated spores.

Results: *Plc*-null spores germinate at alkaline pH, reduced temperature or increased osmolarity, conditions at which the emerging amoebae can not grow. In contrast, food-activated wild-type spores return to dormancy till conditions in the environment allow growth. The analysis of inositol 1,4,5-trisphosphate (IP_3) levels and the effect of added IP_3 uncover an unexpected mechanism how PLC regulates spore germination: i) deletion of PLC induces the enhanced activity of an IP_5 phosphatase leading to high IP_3 levels in *plc*-null cells; ii) in wild-type spores unfavourable conditions inhibit PLC leading to a reduction of IP_3 levels; addition of exogenous IP_3 to wild-type spores induces germination at unfavourable conditions; iii) in *plc*-null spores IP_3 levels remain high, also at unfavourable environmental conditions.

Conclusions: The results imply that environmental conditions regulate PLC activity and that IP_3 induces spore germination; the uncontrolled germination of *plc*-null spores is not due to a lack of PLC activity but to the constitutive activation of an alternative IP_3-forming pathway.

Background

Many extracellular signals activate inositide-specific phospholipase C (PLC) thereby producing the second messengers $Ins(1,4,5)P_3$ and diacylglycerol [1]. Three types of PLC are known, PLC-β, PLC-γ and PLC-δ which are regulated by G-proteins, receptor tyrosine kinases, and Ca^{2+}, respectively [2]. Animals such as human and rat, but also *C. elegans, Artemia, Loligo forbesi* and *Drosophila* possess all three PLC isoforms [3–6]. However, in non-animals exclusively PLC-δ has been identified, e.g. in soybean [7] and catfish [8], and the lower eukaryotes *Dictyostelium discoideum*[9], *Saccharomyces cerevisiae*[10–12] and *Schizosaccharomyces pombe*[13]. This phylogenetic distribution of PLC isozymes is in accordance with the deduced three dimensional structure, suggesting that PLC-δ is the ancient isoform to which specific domains were added in PLC-β and PLC-γ [14,15].

Dictyostelium cells live in the soil where they feed on bacteria. When the bacteria become scarce, starvation induces the expression of a cAMP sensory system that mediates cell aggregation. A fruiting body is formed consisting of spores embedded in a slime droplet on top of a stalk. When nutrients are available, spores germinate

and amoebae search for bacteria by chemotaxis. Spores are relatively safe to environmental stress; no intake of food is required. Furthermore, spores resist extreme temperatures, humidity and pH, and they can pass the digestive track of birds and nematodes [16].

Previously, a *Dictyostelium* mutant was constructed in which the single PLC-δ gene was disrupted resulting in cells without detectable PLC activity [17]. Besides the unexpected finding that these cells contained near-normal IP_3 levels due to an alternative route of IP_3 synthesis [18], they also showed no abnormal phenotype. At optimal laboratory conditions neither growth nor development were affected [17]. However, optimal conditions are not likely to occur for a very long period in the habitat of *Dictyostelium*, which is the upper layer of the soil. Therefore we analyzed the survival of the species at suboptimal conditions.

Results

Normal stress resistance in plc-null cells

A *Dictyostelium* cell lacking the single PLCδ gene shows no abberrant phenotype at laboratory conditions. Therefore we measured the kinetics and dose-dependencies for survival of the amoebae at various stress conditions, including temperature, pH, osmolarity, and removal of extracellular Ca^{2+}. We never observed a significant differences between *plc*-null and control cells (data not shown), suggesting that Dd-PLCδ is not essential to protect the amoebae. This is in strong contrast to another second messenger enzyme, guanylyl cyclase, which protects *Dictyostelium* cells against osmotic stress [19].

Normal differentiation in plc-null cells

A common theme in species that have only the PLCδ isoform is the formation of spores or seeds, which can survive extreme environmental conditions. Sporulation to survive stress comes to an evolutionary advantage when germination of spores occurs only at conditions that allow growth of the emerging organism. We investigated the role of PLC in sporulation and germination. During *Dictyostelium* development about two-thirds of the cells differentiates into viable spores, whereas one-third develops into dead stalk cells. We observed that the proportioning of stalk and spore cells is not different in *plc*-null fruiting bodies (data not shown). Next, *plc*-null cells with a Neo marker were mixed 1:1 with wild-type cells containing a Bsr marker conferring resistance to the antibiotics G418 and blasticidin, respectively. Spores were isolated from the resulting fruiting bodies, and inoculated in microtiter plates at low density such that about 40% of the cultures showed growth. Subsequently cultures were transferred to media with G418 or blasticidin, allowing growth of *plc*-null and wild-type cells, respectively. In three independent experiments we observed that from a total of 101 cultures, 29 did grow in G418, 31 grew in blasticidin, whereas 41 showed growth in both G418 and blasticidin. This experiment demonstrates that the spores have essentially the same *plc*-null/wild-type ratio as the 1:1 mixture of cells from which they were derived, suggesting that Dd-PLCδ does not play a role in the formation of spores.

Aberrant spore germination in plc-null cells

Germination of spores is strictly controlled by environmental factors such as pH, osmolarity and temperature [21]. Figure 1 shows that the germination rate is nearly identical for wild-type and *plc*-null spores at 22°C (half times 4.0 ± 0.2 h and 4.2 ± 0.2 h, respectively). Germination at 16°C is reduced considerably for wild-type spores as compared to 22°C (half-time 17.8 ± 2.6 h). In contrast, *plc*-null spores germinate with almost the same rate at 16°C as at 22°C (half-time 6.6 ± 0.5 h). Similar data were obtained at other temperatures (Table 1). Thus, whereas at 22°C both strains germinate at the same rate, at 13°C *plc*-null spores germinate 15-fold faster than wild-type spores.

Osmotic pressure and pH of the medium are other environmental factors known to affect spore germination. Germination of wild-type spores is more strongly inhibited by sucrose than germination of *plc*-null spores (Figure 1B). Addition of 0.3 M sucrose inhibits wild-type germination 7-fold while germination of *plc*-null spores is retarded only 2-fold (Table 1). At acidic pH, spore germination is inhibited about 25-fold at pH 4.7, 5-fold at pH 5.2, and about 2-fold at the mildly acidic pH of 5.5 (Table 1); no statistical significant differences can be observed between germination of wild-type and *plc*-null spores. At a slightly alkaline pH up to pH 7.8 spore germination is not strongly affected if compared to the optimal pH, but at more alkaline pH germination is inhibited. As with temperature and osmolarity, germination is significantly more affected for wild-type than for *plc*-null spores.

To evaluate the effect of PLCδ deletion on survival of the *Dictyostelium* species, we tested the growth rate of amoebae at stress conditions that influence spore germination. Amoebae grown under optimal conditions were incubated in growth medium at reduced temperature, alkaline or acid pH, or increased osmolarity. The results (Figure 2) show that growth of the amoebae was hardly affected at pH 5.2. In contrast, cells grew about 5-fold slower at 15°C compared to 22°C, while cells did not grow at pH 8.7 or in 0.2 M sucrose. The data reveal no differences in growth rates between wild-type and *plc*-null cells. A role of Dd-PLCδ in spore germination is consistent with the expression of the Dd-PLC gene, which is ab-

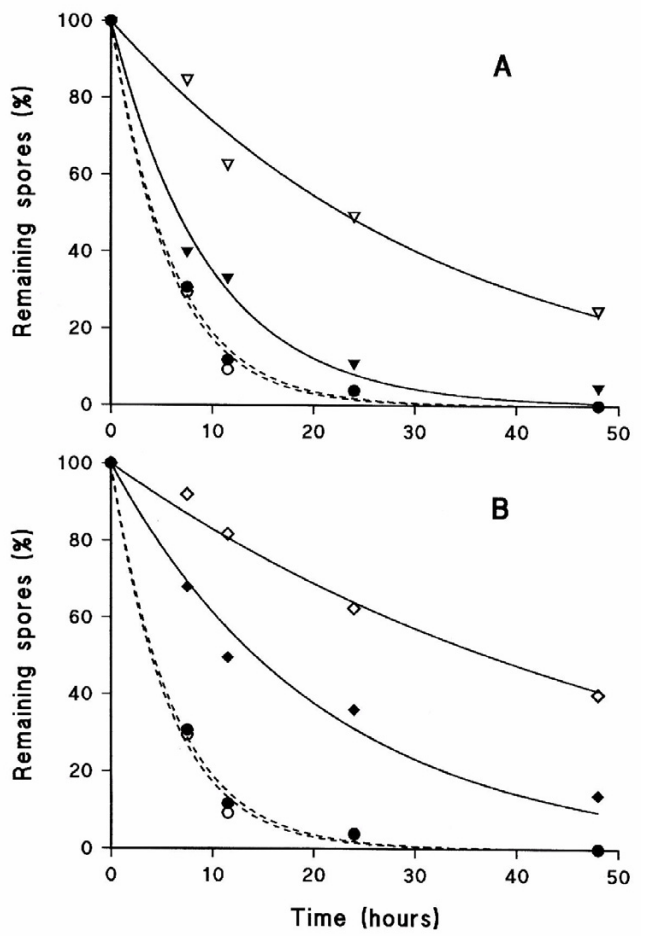

Figure 1
The effect of temperature (A) and osmotic pressure (B) on spore germination. *Plc*-null (closed symbols) and wild-type control (open symbols) spores were inoculated in growth medium at 22°C (circles) or 16°C (triangles), or in growth medium containing 0.2 M sucrose (diamonds). The time at which 50% of the spores were germinated was calculated by exponential curve fitting using the program FigP. The data shown are from a typical experiment; the calculated germination time for many different conditions are summarized in table 1.

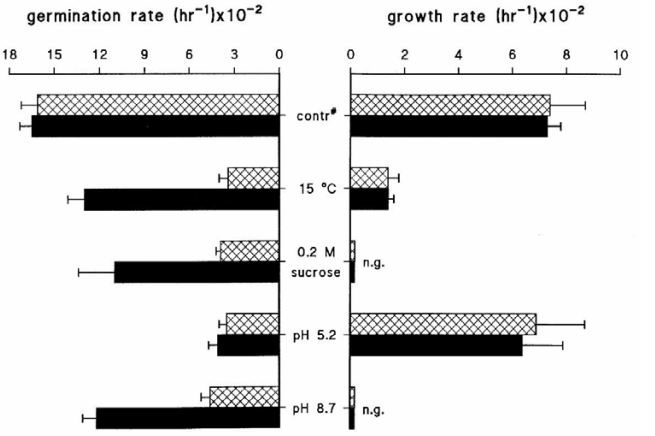

Figure 2
The effect of stress on growth and spore germination. *Plc*-null (filled bars) and wild-type control (hatched bars) spores (left panel) or amoebae (right panel) were inoculated in medium at different conditions as indicated. Germination and growth rate were measured, repectively, which are the first order rate constants for germination and growth, respectively. The results shown are the average and standard deviations for three experiments. The control# refers to medium at pH 7.1 incubated at 22°C; n.g., no growth was observed, the bars indicate the maximal growth rate.

sent in the multicellular slug, but reappears to high levels during culmination and formation of spores [9].

IP_3 levels during spore germination

A simple model can explain the effect of PLC disruption on spore germination: unfavourable conditions activate PLC resulting in a return to dormancy of food-activated spores; in *plc*-null cells PLC can not be activated, so activated spores can not return to dormancy. However, we have observed that amoebae lacking PLC activity have near-normal IP_3 levels due to an alternative route of IP_3 formation, obtained from the degradation of IP_5[18]. Therefore, we have measured IP_3 levels in wild-type and *plc*-null spores germinating at 22°C and 16°C.

The IP_3 content of *plc*-null spores is significantly 50% higher than the IP_3 content of wild-type spores (figure 3). The identity of IP_3 as the Ins(1,4,5)P_3 isomer was confirmed using its sensitivity to degradation by specific enzymes (see [18]; data not shown). The IP_3 concentration of wild-type spores does not change much during germination at 22°C. However, at 16°C the IP_3 levels decrease 53% at three hours after incubating the spores in growth medium. Subsequently, IP_3 levels return to basal levels, which coincides with the return of activated spores to dormancy. In *plc*-null spores no significant alteration of the IP_3 concentration occurs during incubation of spores at 22°C or 16°C. These results suggest that unfavourable conditions in wild-type cells induces the inhibition of PLC activity, and that high IP_3 levels are essential to complete germination.

The observation that IP_3 levels decline in wild-type spores at 16°C, but remain constant in *plc*-null cells, predicts that addition of exogenous IP_3 will induce spore germination of wild-type cells at 16°C. Figure 4 describes the effect of IP_3 on germination of wild-type and *plc*-null spores at 22°C and 16°C. At a concentration of 100 μM, IP_3 significantly promotes the germination of wild-type spores at 16°C; it has no effect at 22°C or on the germina-

Table 1: Spore germination at different environmental conditions

Condition	50% germination (hours)		Significance
	wild-type	*plc*-null	
Temperature (°C)			
10	>250	16.9 ± 0.6	-
13	38 ± 7	7.1 ± 2.0	**
16	17.4 ± 2.6	5.1 ± 0.4	**
19	7.0 ± 0.5	4.3 ± 1.1	*
22 #	4.3 ± 0.3	4.2 ± 0.2	NS
Osmolarity (M)			
0 #	4.3 ± 0.3	4.2 ± 0.2	NS
0.10	5.5 ± 1.1	4.6 ± 0.4	NS
0.15	10.0 ± 0.7	5.2 ± 0.9	**
0.20	17.8 ± 1.2	6.3 ± 1.4	**
0.30	30.9 ± 3.1	9.0 ± 1.0	**
pH			
3.0	>250	>250	-
4.0	>250	>250	-
4.7	127 ± 21	88 ± 18	NS
5.2	19.7 ± 2.6	16.8 ± 2.6	NS
5.5	7.6 ± 2.4	9.4 ± 3.1	NS
6.2	3.6 ± 1.6	4.2 ± 1.7	NS
7.1 #	4.3 ± 0.3	4.2 ± 0.2	NS
7.5	5.2 ±1.5	4.6 ± 1.4	NS
7.8	5.6 ±1.0	5.0 ± 1.3	NS
8.4	8.2 ± 0.9	5.7 ± 0.8	*
9.0	20.6 ± 3.4	9.0 ± 1.1	**
11	>250	>250	-

Spore germination was measured at different temperatures, increased osmolatity by added glucose or at different pH of the medium; #, normal medium. Data are the means and standerd deviations from three independent experiments. NS, the difference between wild-type and *plc*-null is not significant at $P > 0.05$; * and **, significant at $P < 0.05$ and $P < 0.01$, respectively.

tion of *plc*-null spores. The effect of IP_3 is specific for the active isomer Ins(1,4,5)P_3, because Ins(1,3,4)P_3 and inositol have no effect at this concentration.

Discussion

The present and previous results suggest that PLCδ has no essential function in cell growth, chemotaxis and differentiation in *Dictyostelium*, but appears to play an essential function during spore germination, which is a complex sequence of events. In the sporehead, germination is inhibited by high osmolarity mediated by a specific adenylyl cyclase ACG [22]. The activation of spores by nutrients is followed by a lag phase. Subsequently spores swell and amoebae emerge. Once a spore has swollen, germination becomes irreversible, but during the lag phase activated spores can return to dormancy [23]. Several environmental conditions that are harmful to amoebae, but to which spores are resistant, are known to induce dormancy (figure 5). Our observations imply that without environmental stress, *plc*-null and control spores germinate at the same rate. However, *plc*-null spore germinate at 16°C, pH 8.7 and 0.2 M sucrose, conditions that inhibit growth of the emerging amoebae. Inhibition of spore germination by high osmolarity is probably a dual control by ACG and PLC.

A simple biochemical hypothesis could explain the results: unfavourable conditions activate PLC resulting in IP_3 formation that inhibits germination; *plc*-null cells lack the ability to synthesize IP_3 by which activated spores can not return to dormancy. This hypothesis appears to be incorrect. Firstly, IP_3 levels of *plc*-null spores

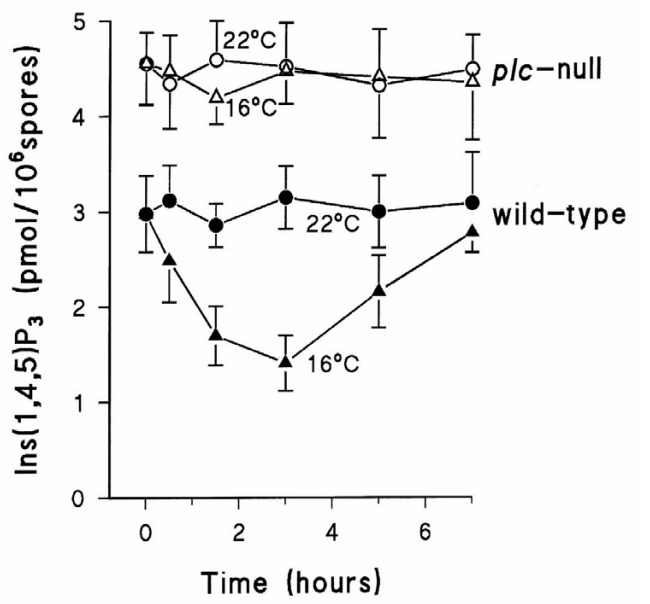

Figure 3
IP_3 formation in germinating spores. Wild-type and *plc*-null spores were incubated in growth medium at 22°C or 16°C. At the times indicated samples were withdrawn and assayed for the IP_3 content using an isotope dillution assay. The means and standard deviations of triplicate determinations from two independent experiments are shown.

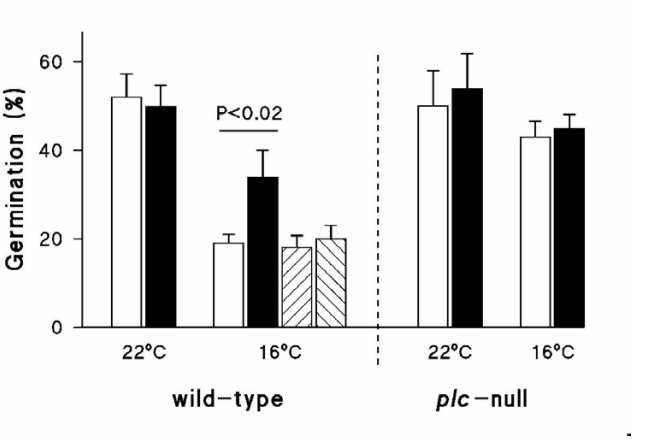

Figure 4
The effect of IP_3 on spore germination. Wild-type and *plc*-null spores were incubated in growth medium at 22°C or 16°C in the absence (open bars) or presence of 100 μM Ins(1,4,5)P_3 (filled bars); the experiment of wild-type cells at 16°C includes two controls (hached bars) with 100 μM inositol and Ins(1,3,4,)P_3, respectively. The fraction of germinated spores was determined after 7 hours. The means and standard deviations of triplicate determinations from two independent experiments are shown. The effects of additives are not significant except for Ins(1,4,5)P_3 at 16°C in wild-type cells.

are 50% higher than IP_3 levels of wild-type spores. Secondly, in wild-type cells unfavourable conditions inhibit rather than stimulate IP_3 formation. Thirdly, at unfavourable conditions exogenously added IP_3 does not inhibit germination of *plc*-null spores, but promote germination of wild-type spores. Finally, Lydan and Cotter [24] have demonstrated that in wild-type cells IP_3 acts synergistically with autoactivator to stimulate germination of saponin treated spores, and that EGTA will inhibit swelling of autoactivating spores.

Dictyostelium as well as mammalian cells contain two routes for IP_3 formation, PLC-mediated hydrolysis of the phospholipid PIP_2, and degradation of a specific IP_5-isomer, Ins(1,3,4,5,6)P_5, by the enzyme MIPP. Non-equilibrium labelling experiments with [3H]inositol demonstrate that in wild-type *Dictyostelium* cells at least 90% of IP_3 is produced by PLC, whereas in *plc*-null cells all IP_3 is derived from IP_5[18,25]. In addition, our observation of depleted IP_5 levels in *plc*-null cells, strongly suggesting that the IP_3 pathway from IP_5 is activated in *plc*-null cells [17].

All experiments are consistent with a more complex hypothesis for the regulation of spore germination in *Dictyostelium* (Figure 5). *Plc*-null spores contain an activated route of IP_3 formation from IP_5, leading to enhanced IP_3 levels in comparison to wild-type spores. Activated spores will germinate when IP_3 levels remain high, but return to dormancy at reduced IP_3 levels. Unfavourable conditions inhibit PLC leading to reduced IP_3 levels in food-activated wild-type spores. Apparently IP_3 formation from IP_5 is not inhibited, IP_3 levels do not decrease, and spore germination continues at unfavourable conditions in *plc*-null spores. Finally, exogenously added IP_3 promotes germination of wild-type spores at conditions that induce a depletion of intracellular IP_3 and a return to dormancy. The depletion of intracellular IP_3 in wild-type cells at reduced temperature is transient. Thus, spore germination is inhibited although IP_3 levels have returned to the high basal levels. Apparently, the transient reduction of IP_3 levels during spore activation has modified the sporulation proces.

Many eukaryotes, including plants and fungi make spores that resist severe environmental stress. These spores allow species to preserve their genes even when the individuals that have produced the spores are dead. In contrast, mammals can only transmit their genes while the individual is still living. Gene maintenance through spores requires strict regulation of spore germination, which should only occur when the environment can support growth of the emerging organism. It is clear

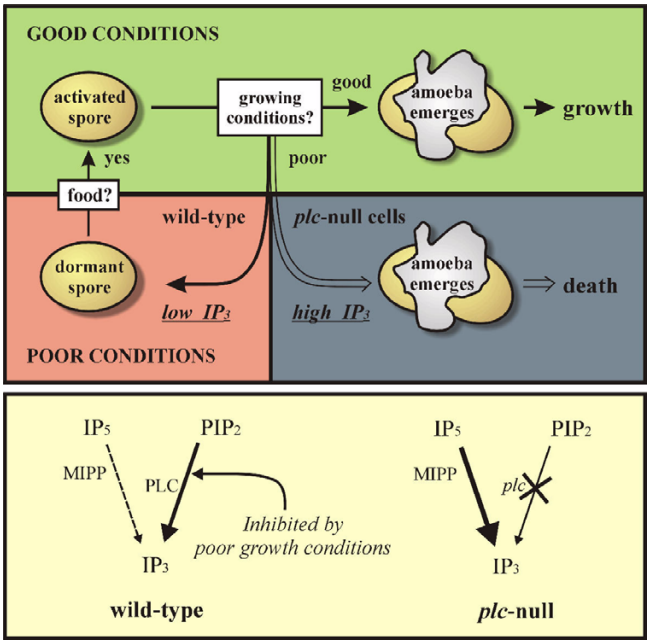

Figure 5
The function of Dd-PLC-δ during spore germination
The figure shows fate decisions that are made during the germination of *Dictyostelium* spores. Food triggers spore germination. However, before an amoeba emerges, environmental conditions that can not support growth induce the activated spore to return to dormancy. Deletion of Dd-PLCδ causes the activated spore to germinate at these unfavourable conditions, leading to death of the emerging amoeba. For spore germination high IP_3 levels are essential. In *Dictyostelium* IP_3 can be formed via two routes, PLC acting on PIP_2 and MIPP acting on IP_5. Cells lacking PLC possess high IP_3 levels due to the enhanced activity of MIPP. Unfavourable conditions for spore germination inhibit PLC activity, leading to a reduction of IP_3 levels in wild-type cells, but unfavourable conditions have no effect on MIPP by which IP_3 levels remain high in *plc*-null cells.

that a return to dormancy of food activated spores at environmental conditions that can not support growth of the emerging organism is of great evolutionary importance. The lack of Dd-PLCδ is a severe disadvantage in the wild, even though under normal laboratory conditions no phenotype of *plc*-null cells can be observed. It is not known whether this function of PLCδ in *Dictyostelium* is restricted to spore-forming organisms possessing only the δ-isoform of PLC, and that in higher organisms PLCδ fulfils a similar control role, distinct from the functions of the additional PLCβ and PLCγ.

Conclusions

By analyzing spore germination and IP_3 levels in wild-type and *plc*-null strains we conclude that harmful environmental conditions inhibit PLC activity and that the reduced IP_3 levels prevent spore germination. In *plc*-null strains, an alternative pathway for IP_3 formation is induced that is not inhibited by harmful environmental conditions. As a consequence, IP_3 levels are not inhibited and *plc*-null spores germinate at environmental conditions where the emerging amoebae can not survive.

Materials and methods

Strains

Two sets of *Dictyostelium* cells were used. HD10 (*plc*-null cells) and HD11 (wild-type control for HD10) are G418 resistant clones in an AX3 background [17]. Clone 1.19 (*plc*-null cells) and 0-mut (PLC expressed in 1.19 using an actin 15 promotor) are transformants in a DH1 background [26]. The experiments presented in this report were performed with the combination HD10/HD11; the experiments of figures 1 and 2 were reproduced with the combination 1.19/0-mut.

Spore germination and cell growth

Spores were isolated from 1 to 3 days old fruiting bodies. After treatment with 0.5% NP-40 for 3 minutes to kill remaining amoebae, spores were washed three times with water, and inoculated at a final density of 1.5×10^6 spores per ml in flasks containing 10 ml medium as indicated. The flasks were shaken (150 rpm) at 22°C or 16°C. At various time intervals the spores were observed by phase contrast microscopy and scored for unswollen spores and amoebae. Germination is defined as the fraction of spores that have emerged as amoebae.

Cell growth was measured in medium as indicated in figure 2 using freshly growing cells from a culture at 22°C in HG5 medium at a density of 2×10^6 cells/ml.

Determination of IP_3 levels

Two days old spores were incubated at a density of 1.5×10^8/ml in HG5 medium at 22°C or 16°C. At the times indicated in figure 3, 20 µl of the suspension was added to 20 µl ice-cold 3.5% (v/v) perchloric acid. After incubation in a sonication bath for 15 min, the lysates were neutralized with 10 µl $KHCO_3$ (50% saturated at 22°C). IP_3 levels were measured in the extracts using an isotope dilution assay [27].

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